REMARKS

Claims 1-7, 9-12, 25-28, and 48-54 were pending in the subject application. Applicants hereinabove have amended claims 1, 9, 12, 25, 48, 52, and 53 and canceled claims 6, 49, and 50 without disclaimer or prejudice to applicants' right to pursue the subject matter of these claims in the future. Support for the amendments to these claims may be found, *inter alia*, in the specification as follows: Claim 1: page 13, lines 28-30; page 10, lines 19-24; page 39, lines 18-20; page 24, lines 25-30; page 11, lines 1-3; and Figure 1. Claim 9: page 19, lines 17-18; Claim 25: page 6, lines 19-31; page 12, lines 9-22 and Figure 19; Claim 53: page 39, lines 18-20; page 24, lines 25-30, page 11, lines 1-3 and Figure 1. Applicants note that claims 48 and 52 have been revised to correct a grammatical error. Upon entry of this Amendment, claims 1-5, 7, 9-12, 25-28, 48, and 51-54, as amended, will be pending and under examination.

1. Specification, 35 USC §102 ; 35 USC §112

Applicants note that the Examiner has withdrawn the objection previously made to claims 11 and 12.

Applicants also note that the Examiner has withdrawn the rejections previously made under 35 USC §101 and 35 USC §112.

2. Claim Rejection under 35 USC §112, Second Paragraph

The Examiner has rejected Claims 1-7, 9-12, 26-28, 48, 51 and 52 as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention, because it is unclear what the "0.05%" recited in part vi) of claim 1 is relative to.

In response, applicants respectfully traverse the Examiner's ground of rejection. Nevertheless, without conceding the correctness of the

Examiner's rejection, but to advance prosecution, applicants note that claim 1 has been amended hereinabove.

Specifically, part vi) of claim 1 has been amended to recite "a degree of senescence of less than 0.05% after 60 population doublings". From this phrasing, it is clear that "0.05%" refers to the percentage of cells which are senescent after the claimed cell has undergone 60 population doublings.

Accordingly, applicants submit that claim 1, as amended, and claims 2-5, 9-12, 26-28, 48, and 51 and 52 which depend from claim 1, comply with the requirements of 35 USC §112.

In view of these remarks, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection under 35 U.S.C. §112, second paragraph.

3. Claim Rejections 35 USC §103

In response to the Examiner's objections under 35 USC §103, Applicant provides the following comments:

Claims 9-12, 25-28, 48-50, 53 and 54

The Examiner rejected claims 9-12, 25-28, 48-50, 53 and 54 under 35 USC 103(a) as being unpatentable over Katz et al (WO 00/53795, 2000) in view of Akanbi et al. (1994), Hedrick et al. (US 2003/0082152, published May 1st, 2003) and Haynesworth et al. (US 5,733, 542, 1998).

The Examiner stated that Katz et al. teach human lipo-derived stem cells which can differentiate into adipocytes, osteocytes, myocytes or chondrocytes and whose telomerase activity was similar to that exhibited by previously reported human stem cells. The Examiner

acknowledged on page 5 of the Office Action that Katz et al. do not teach that the adipose tissue is obtained from children under 10 as it is obtained from liposuction, which implies the tissue is from adults. The Examiner, however, stated that Akanbi et al. teach that adipose precursor cells from young animals replicate faster than cells from older animals and/or contain more clones capable of full differentiation into adipocytes. The Examiner therefore maintains it would have been obvious to one of ordinary skill in the art to obtain stem cells from adipose tissue of children using the teachings of Katz et al. and Akanbi et al.

The Examiner also acknowledged that neither the cells disclosed in Katz et al. nor those disclosed in Akanbi et al. are shown to have the characteristics of claim 1, but stated that the method of obtaining the stem cells disclosed in Katz et al. is the same as the claimed invention, and that therefore the cells obtained from children would have these characteristics.

The Examiner also stated that the teaching of Hedrick et al. is readable on the steps of "enriching the CA population" and "inducing proliferation of CA stem cells" of claim 25 as Hedrick et al.'s population is enriched by PBS washing and by keeping the cells confluent to maintain their multipotency.

The Examiner finally stated that Haynesworth et al. teach plating cells for 3 days before removing non-adherent stem cells, and thus that the skilled artisan using the teachings of Katz et al., Akanbi et al. and Haynesworth et al., would have arrived at the "CS" cells of claims 49 and 50.

In response, Applicants respectfully traverse the Examiner's ground of rejection. In addition, Applicants note the following:

1. **Akanbi et al. disclose neither "stem cells", nor cells obtained from humans.**

Akanbi et al. teach that newborn pigs have more preadipocytes capable of attaching and differentiating into mature adipocytes than 7 month old pigs. Akanbi et al. thus teach that adipocyte precursor cells from very young animals may replicate faster than those of older animals, and that adipose tissue from young animals may contain more clones capable of full differentiation into adipocyes. **This teaches away from the invention.**

Indeed, **Akanbi et al. do not provide any teaching at all relative to stem cells, let alone human stem cells.** Pre-adipocytes are a completely different type of cells than stem cells, in particular because they are at a more advanced stage of differentiation and can only differentiate into adipocytes. Stem cells on the other hand are in an undifferentiated state and can differentiate into many different cell types, as shown in the present application.

Moreover, stem cells proliferate much more slowly than other cell types (see page 10, lines 2-4 of the specification). The skilled man would thus not use a cell composition which proliferates rapidly (i.e. from young pigs), because this means it is very rich in fastly dividing cells, that is, cells which are not stem cells.

Thus the teaching of Akanbi et al. is not relevant because it relates to cells which are very different from stem cells in general and even more so from human stem cells.

2. Hedrick et al. do not teach the separation of cells which are adherent after less than 12 hours.

The separation of "CA" cells which are adherent after **less than 12 hours** from "CS" cells which are adherent after more than 12 hours is an essential feature of the process of preparation of the claimed cells. Indeed, these two cell types have been shown to have some properties in common, such as a capacity to undergo differentiation into a number of cell types, at least at early stages, and an HLA class I negative phenotype, but also others which are different. In particular, CS cells do not enter into quiescence and undergo a more limited number of doublings (see page 9, lines 12-21; page 46, lines 3-25 and figures 5, 6 and 7 of the specification). Contrary to what is stated by the Examiner, this process feature is not disclosed by Hedrick et al.

Hedrick et al. teach that cells are incubated **overnight**, and then that non-adherent red blood cells are eliminated (paragraph 202). However, the term "overnight" is not equivalent to 12 hours. Indeed, this term should be interpreted as corresponding to at least the time-frame starting when the skilled man, performing work during normal working hours, would leave his laboratory, i.e. not later than 6 PM, and ending when he would arrive in the morning, i.e. not earlier than 8 AM. **Therefore, the term "overnight" should be interpreted as a time-frame of at least 14 hours**.

Moreover, it is stated by Hedrick et al. that the non-adherent red blood cells are removed, not that one type of adherent cell is separated from another as is the case in the process for preparing the claimed cells. The skilled man would thus

understand the teaching to be that this step is performed solely to remove red blood cells. As red blood cells do not adhere to plastic, no matter how long they are left in culture, the "overnight" time-frame would thus not be construed as a precise length of time but rather as a minimum length of time which needs to be respected. Moreover, other means of removing red blood cells are known in the art, for example, the use of a lysis buffer.

Hedrick et al. thus teach that red blood cells should be removed from the cell culture, and indicate a means for achieving this, but do not teach the separation of two cell populations which **both** are adherent, and do not provide any guidance as to the importance of performing such a separation step among adherent cells after only 12 hours in culture.

3. **The method of obtaining the stem cells disclosed in Katz et al. is not the same as the method of obtaining the stem cells of the claimed invention**

Indeed, as detailed above, the process described in the specification of the present application for obtaining the claimed stem cells comprises a **separation step** between two cell populations based on the length of time in which they become adherent. This step allows separation of a "CA" cell population containing true stem cells from a "CS" cell population containing precursor cells which as indicated above has some characteristics in common with the "CA" cell population (capacity to differentiate into a number of cell types at an early stage, HLA class I negative phenotype), but are not capable of entering into quiescence nor can undergo 130 population doublings.

Further, this process also comprises a step of enriching the "CA" population until a **quiescent** cell population is obtained, and a step of inducing that quiescent population of stem cells to proliferate.

As indicated in the specification of the present application, quiescence is a state in which the cells neither proliferate nor enter senescence (see specification, page 6, lines 4-5), and the capacity to enter into quiescence is a **characteristic of true stem cells**. The step of enriching the "CA" population until a **quiescent** cell population is obtained thus provides a cell population composed only, or at least essentially, of true stem cells. Indeed, stem cells enter into **quiescence** after 50 to 80 population doublings whereas the other cells types enter **senescence** at that stage or even earlier.

Thus combining the step of separating the "CA" cell population from the "CS" cell population, and the step of enriching the "CA" population until a **quiescent** cell population is obtained allows one to eliminate the cells which are not true stem cells.

Further, in the process disclosed in the specification, the step of inducing the quiescent population of stem cells to proliferate permits an increase in the number of stem cells. Thus, contrary to the process disclosed by Katz et al., this process permits separation of the true stem cells from other precursor cells, and enrichment of the pure stem cell population thus obtained to provide a high number of true stem cells.

Thus, the claimed cells are obtained by a method which allows obtaining of a very 'pure' population of stem cells and also of a very high number of cells, and ensures that the cells obtained

are indeed stem cells because non stem cells cannot enter quiescence.

Katz et al. did not separate a "CA" population of cells from a "CS" population. They did not show that the cells they isolated could enter quiescence and they differentiated these cells at an early stage. As stem cells are a very rare cell type, the cells isolated by Katz et al. would thus be essentially precursor cells such as the "CS" cells disclosed in the invention, not true stem cells such as the "CA" cells.

Therefore, contrary to what is asserted by the Examiner, replacing the adult cells used by Katz et al., with cells obtained from children is not the only change to the method taught by Katz et al. to obtain applicants' claimed cells.

4. The claimed cells have been shown to have a broader differentiation potential than those taught by Katz et al.

Katz et al. teach human lipo-derived stem cells which can differentiate into cell types which belong to the limb bud mesoderm (adipocytes, osteocytes, myocytes and chondrocytes). The cells according to the invention, however, can differentiate not only into limb bud mesoderm cell types (adipocytes, osteocytes, myocytes), but also into a visceral mesoderm cell type (endothelial cells) (see passage bridging pages 15 and 16 of the specificaiton). The cells of the invention thus not only can undergo differentiation into cells of the mesenchymal lineage as the cells disclosed by Katz et al., but can differentiate into cells of the mesodermal lineage. They have thus been shown to have a **broader differentiation potential** than those of Katz et al.

5. **The telomerase activity of the cells taught by Katz et al. is not the same as that of the claimed cells.**

   As already indicated in the Applicant's response to the previous office action (see section 4.a of the remarks section of the amendment submitted in response to February 27, 2007 final office action), the cells of Katz et al. do NOT have an endogenous telomerase activity of at least 20% to 50% of the telomerase activity of the HEK293T transformed cell line and which is maintained over at least 130 population doublings. Thus, **the telomerase activity of stem cells as claimed in the present invention is not the same as that of the precursor cells described in Katz et al.**

6. Claims 49 and 50 relative to "CS" cells and "CS" cell populations have been cancelled.

In conclusion, the method for preparing the stem cells of the invention differs in several essential features from the method of Katz et al., and in particular by

- the use of adipose tissue from children under 10 years of age,
- the separation of the "CA" population from the "CS" population,
- the enriching of the "CA" population until quiescence, and
- the inducing of proliferation after quiescence.

These features are neither taught nor suggested in the prior art. In particular, the publication by Akanbi et al. relates to cells which are not stem cells and are not human cells. Akanbi et al. provide no information on stem cells from humans. The use of the cells taught by Akanbi et al. in the method for producing the claimed cells does not

provide applicants' claimed cells. Hedrick et al. neither teach nor suggest the separation of two cell populations which **both** are adherent, and the removal of non adherent red blood cells after overnight culture is not equivalent to performing such a separation step among adherent cells after 12 hours in culture. Finally, none of the prior art document teaches or suggests that the cells should be cultivated until quiescence, and that after quiescence, proliferation should be induced again. The cells taught in the prior art are not the same as the stem cells according to the invention because (i) the starting material is not the same, and (ii) the method steps are not performed.

Accordingly, applicants maintain that no combination of Katz et al. with Akanbi et al., Hedrick et al. and Haynesworth et al. renders obvious applicants' claimed invention.

It is thus submitted that claims 9-12, 25-28, 48, 53 and 54 are patentable over Katz et al (WO 00/53795, 2000) in view of Akanbi et al. (1994), Hedrick et al. (US 2003/0082152, published May 1st, 2003) and Haynesworth et al. (US 5,733, 542, 1998) and comply with the requirements of 35 USC 103(a). Applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Claims 1-7, 9-12, 26-28, 48, 51 and 52

The Examiner rejected claims 1-7, 9-12, 26-28, 48, 51 and 52 under 35 USC 103(a) as being unpatentable over Katz et al (WO 00/53795, 2000) in view of Akanbi et al. (1994) and West (US 5,589,483, 1996).

The Examiner again stated that the combined teaching of Katz et al. and Akanbi et al. provides guidance to obtain stem cells from the adipose tissue of children.

The Examiner also acknowledged that Katz et al. and Akanbi et al. do not teach that the stem cells exhibit an endogenous beta-galactosidase activity of less than 0.05% at 60 population doublings. The Examiner, however, stated that West teaches that recombinant vectors that comprise the HIV LTR promoter positioned to drive expression of a reporter gene can be used to indicate cell senescence, and thus it would have been obvious to an ordinary artisan to use such an expression vector in stem cells to determine whether the cells were senescent.

In response, Applicants respectfully traverse the Examiner's ground of rejection. In addition, Applicants note:

1. Claim 1 has been amended to recite "**a degree of senescence of less than 0.05% at 60 population doublings**" rather than "an endogenous beta-galactosidase activity of 0.05% after 60 population doublings".

2. **West does not provide a means to obtain non-senescent stem cells**

   It is noted that what is claimed is a cell having a particular level of non-sensecence, not the manner in which non-sensecence is measured. West does not provide a means to obtain cells which have the claimed level of senescence. Indeed, West discloses a means to measure senescence using a recombinant vector in which expression of a reporter gene, e.g. beta-galactosidase is driven by the HIV LTR promoter. West does not teach cells which have a degree of senescence of less than 0.05% at 60 population doublings, nor does he provide any guidance as to how to obtain such cells.

Further, contrary to what appears to have been understood by the Examiner, according to the invention, the measured level of beta-galactosidase activity is the level of **endogenous** beta-galactosidase activity at **pH 6,** not the level of beta-galactosidase activity driven from a vector as described by West. Indeed, as taught by Dimri et al. (a copy of which is attached hereto as **Exhibit A**), endogenous β-galactosidase activity at pH 6 is a measure of senescence in a cell population. **A level of less than 0.05% shows that at 60 population doublings the population is not senescent, but is rather quiescent**. This means that the cells are capable of undergoing a very high number of doublings without becoming senescent, and is thus an indication of "stemness".

It is also brought to the attention of the Examiner that example 9 (pages 52-53) which he cites relates to the expression of a transgene, such as the LacZ reporter gene, not to the measure of senescence.

The teaching of West is thus irrelevant for the assessment of the non-obviousness of applicants' claimed cells.

3. Further, as previously put forward in item 2 of section 4.1 above, the teaching of Akanbi et al. is not relevant as it discloses neither "stem cells" nor cells obtained from humans.

In conclusion, as previously explained at the end of section 4.1, the method for obtaining the stem cells of the invention differs from the method of Katz et al. in several essential features which are neither taught nor suggested in the prior art. The cells taught in the prior art are thus not the same as the stem cells according to the invention because they are obtained by a different process. The cells of the

invention further have **a degree of senescence of less than 0.05% at 60 population doublings**. This feature is neither taught nor suggested by West. In particular, West does not teach cells which have an endogenous level of beta-galactosidase activity of less that 0.05% at pH 6.

Accordingly, applicants maintain that no combination of Katz et al. with Akanbi et al., and West et al. renders obvious applicants' claimed invention.

It is thus submitted claims 1-7, 9-12, 26-28, 48, 51 and 52 are patentable over Katz et al (WO 00/53795, 2000) in view of Akanbi et al. (1994) and West (US 5,589,483, 1996), and comply with the requirements of 35 USC 103(a). Applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Summary

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee is deemed necessary in connection with the filing of this Amendment. However, if any additional fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,

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I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to:

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Reg. No. 28,678

Applicant: Anne-Marie Rodriguez et al.
Serial No.: 10/632,581
Filed: July 31, 2003
Exhibit A

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Vol. 92, pp. 9363–9367, September 1995
Cell Biology

A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*

(replicative senescence/tumor suppression/β-galactosidase)

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Communicated by Arthur B. Pardee, Dana–Farber Cancer Institute, Boston, MA, June 12, 1995 (received for review March 28, 1995)

ABSTRACT **Normal somatic cells invariably enter a state of irreversibly arrested growth and altered function after a finite number of divisions. This process, termed replicative senescence, is thought to be a tumor-suppressive mechanism and an underlying cause of aging. There is ample evidence that escape from senescence, or immortality, is important for malignant transformation. By contrast, the role of replicative senescence in organismic aging is controversial. Studies on cells cultured from donors of different ages, genetic backgrounds, or species suggest that senescence occurs *in vivo* and that organismic lifespan and cell replicative lifespan are under common genetic control. However, senescent cells cannot be distinguished from quiescent or terminally differentiated cells in tissues. Thus, evidence that senescent cells exist and accumulate with age *in vivo* is lacking. We show that several human cells express a β-galactosidase, histochemically detectable at pH 6, upon senescence in culture. This marker was expressed by senescent, but not presenescent, fibroblasts and keratinocytes but was absent from quiescent fibroblasts and terminally differentiated keratinocytes. It was also absent from immortal cells but was induced by genetic manipulations that reversed immortality. In skin samples from human donors of different age, there was an age-dependent increase in this marker in dermal fibroblasts and epidermal keratinocytes. This marker provides *in situ* evidence that senescent cells may exist and accumulate with age *in vivo*.**

Normal, somatic cells do not divide indefinitely. This property, termed the finite replicative lifespan of cells, leads to an eventual arrest of cell division by a process termed replicative or cell senescence. Replicative senescence is generally studied in culture, where cells can be grown and monitored—in a controlled fashion—until most or all cells in a population senesce (1–4). Indirect evidence suggests that replicative senescence occurs *in vivo* (see refs. 2 and 4).

There are several salient features of senescence in culture (2–4). (*i*) It is exceedingly stringent in human cells, which, unlike many rodent cells, rarely if ever spontaneously immortalize. (*ii*) Senescent cells arrest growth with a G_1 DNA content and do not enter S phase in response to physiologic mitogens. Many genes remain mitogen-inducible, but some that are needed for cell cycle progression are repressed. (*iii*) Multiple, dominant-acting genes control the finite replicative lifespan and growth arrest of senescent cells. (*iv*) Senescent cells show selected, cell-specific changes in function. (*v*) Senescent cells remain metabolically active, and resist apoptotic death, for long periods of time.

The idea that cellular senescence is tumor suppressive stems from molecular, cellular, and *in vivo* data. Immortality greatly increases the susceptibility to malignant transformation, in culture and *in vivo* (5, 6). Indeed, many tumors contain immortal cells or cells with an extended replicative lifespan (7). In addition, some oncogenes act primarily to immortalize or extend the lifespan of cells. Finally, Rb and p53, tumor suppressors that commonly suffer loss-of-function mutations in human cancers, are critical for cell senescence (see refs. 4 and 8).

The idea that senescence contributes to aging, by contrast, stems largely from studies in culture. Cells cultured from old donors tend to senesce after fewer population doublings (PD) than cells from young donors (9–11). Thus, cells in renewable tissues may deplete their replicative potential during aging. Moreover, cells from short-lived species tend to senesce after fewer PD than cells from long-lived species (11, 12), and cells from humans with hereditary premature aging syndromes senesce more rapidly than age-matched controls (13, 14). Thus, cellular replicative lifespan and organismic lifespan may be under overlapping genetic control. Together, the data support the idea that replicative senescence occurs *in vivo*. It is assumed that senescent cells accumulate *in vivo*, where their altered phenotype contributes to age-related pathology.

Senescent cells are identified in culture by their failure to synthesize DNA under optimal conditions and exponential increase with passage. *In vivo*, however, cell growth is not easily manipulated or monitored, and DNA synthesis measurements do not distinguish senescent cells from quiescent or terminally differentiated cells. Senescence markers identified in culture (2–4) either are not specific or require single cell quantitation or manipulations (e.g., mitogen stimulation) that are difficult to do or control *in vivo*. Thus, the idea that senescent cells accumulate with age has remained speculative because senescent cells cannot be identified in tissues.

We describe a simple biomarker for replicative senescence that provides *in situ* evidence that senescent cells may persist and accumulate with age *in vivo*.

MATERIALS AND METHODS

Cells. WI-38, AG09602, AG06234, AG07720, AG0439, AG00780, and AG06300 were from the Coriell Institute (Camden, NJ). HCA2, HT1080, HeLa, TE85, and CMV-MJ were from O.P.-S., and 88-6 and MC17326 were from J. Oshima and G. Martin (University of Washington, Seattle).

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Abbreviations: PD, population doublings; NHEK, neonatal human epidermal keratinocytes; dex, dexamethasone; β-Gal, β-galactosidase; SA-β-Gal, senescence-associated β-Gal.

**To whom reprint requests should be addressed.

Cells were cultured, as described (15–17). Neonatal human epidermal keratinocytes (NHEK; Clonetics, San Diego) were cultured as per the supplier's instructions in 10% CO_2 and 10 mM Hepes (pH 7.4); differentiation was induced by $CaCl_2$ or phorbol 12-myristate 13-acetate (18). Microcell fusion was performed, as described (16, 17). IDH4 cells were grown in 1 μM dexamethasone (dex) or arrested in 10% charcoal-stripped serum and medium lacking phenol red and dex, as described (19). Human endothelial cells (strain H3605) were from J. Wessendorf and T. Maciag (American Red Cross, Rockville, MD) (20), neonatal melanocytes were from Z. Abdel-Malek (University of Cincinnati), adult melanocytes were from shave biopsies (21), mammary cells were from V. Band (New England Medical Center, Boston) (22), and ovarian cells were from N. Auersperg (23). SV40-WI38, C33a, U2OS, SAOS, and HTB9 were from the American Type Culture Collection.

[^{3}H]Thymidine Labeling. Sparse cells (1–5 × 10^3 per cm^2) were given 10 μCi of [^{3}H]thymidine (60–80 Ci/mmol; 1 Ci = 37 GBq) per ml for 48–72 hr, stained where indicated, washed in phosphate-buffered saline (PBS), rinsed twice in methanol, and processed for autoradiography, as described (15).

β-Galactosidase (β-Gal) Staining. Cells were washed in PBS, fixed for 3–5 min (room temperature) in 2% formaldehyde/0.2% glutaraldehyde (or 3% formaldehyde), washed, and incubated at 37°C (no CO_2) with fresh senescence-associated β-Gal (SA-β-Gal) stain solution: 1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) per ml (stock = 20 mg of dimethylformamide per ml)/40 mM citric acid/sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM $MgCl_2$. Staining was evident in 2–4 hr and maximal in 12–16 hr. To detect lysosomal β-Gal, the citric acid/sodium phosphate was pH 4.0.

Skin Samples. Human skin from individuals undergoing Moh's micrographic surgery for skin cancer was rapidly frozen in liquid nitrogen, and mounted in OCT. Thin sections (4 μm) were cut, mounted onto glass slides, fixed in 1% formalin in PBS for 1 min at room temperature, washed in PBS, immersed overnight in SA-β-Gal staining solution, counterstained with eosin, and viewed under bright field at 100–200× magnification.

RESULTS

β-Gal in Cultured Human Fibroblasts. Senescent human fibroblasts expressed a β-Gal that was detected in single cells by X-Gal, which forms a local blue precipitate upon cleavage (24), independent of DNA synthesis measurements. Early, middle, and late passage cultures (15) were given [^{3}H]thymidine to label presenescent cells, stained for β-Gal, and processed for autoradiography. Thus, individual cells were monitored simultaneously for ability to synthesize DNA and β-Gal activity. Two human fibroblast strains, HCA2 (neonatal foreskin) and WI-38 (fetal lung), showed similar results.

Most cells express a lysosomal β-Gal that is optimally active at about pH 4 (25). Indeed, presenescent and senescent cells stained equally well when assayed at pH 4 (Fig. 1 *A* and *B*). Neither stained at pH 7.5, the optimum for the bacterial β-Gal reporter enzyme (not shown). At pH 6, only senescent cells stained (Fig. 1 *C* and *D*). We refer to this pH 6 activity as the SA-β-Gal.

Most early passage cells were labeled with [^{3}H]thymidine and did not express SA-β-Gal (Fig. 1*C*). The occasional SA-β-Gal-positive cell almost invariably was unlabeled (Fig. 1*C*). With increasing PD, there was a striking inverse relationship between SA-β-Gal staining and radiolabeling (Fig. 2*A*). By late passage, most cells were unlabeled and strongly SA-β-Gal positive (Fig. 1*D*). The most intense staining was perinuclear and in late passage cultures (Fig. 1*D*). Upon replating, senescent cells retained SA-β-Gal and did not divide (not shown). Typically, radiolabeled cells were SA-β-Gal negative, and unlabeled cells were SA-β-Gal positive (see legend to Fig. 1).

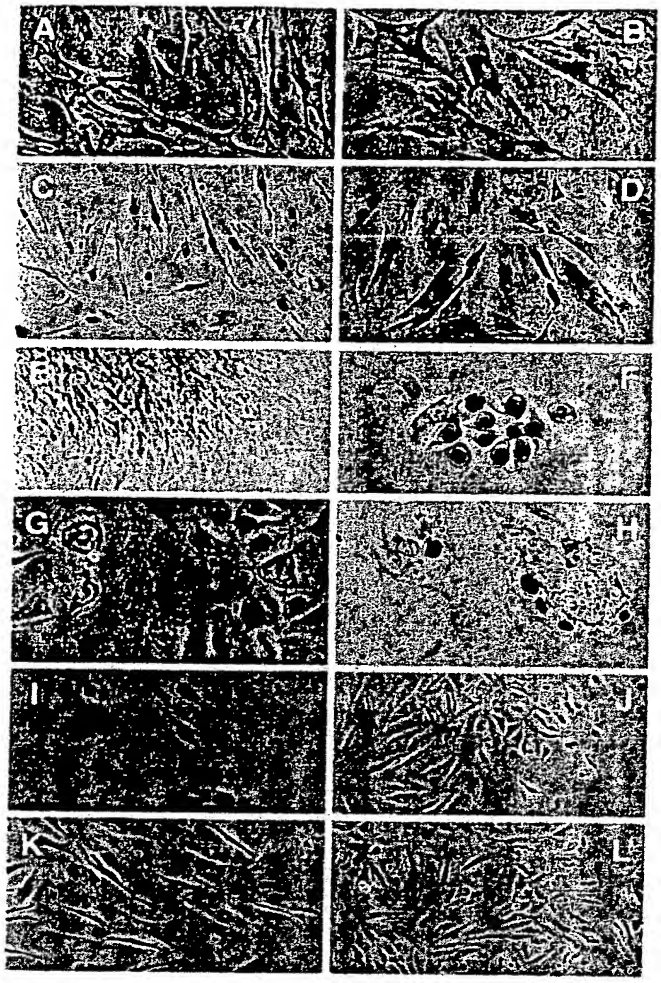

FIG. 1. β-Gal in cultured human cells. Cells were radiolabeled, stained, and photographed at 100× (*A–E* and *J–L*) or 200× (*F–I*) magnification (final magnifications: *A–E* and *J–L*, ×60; *F–I*, ×120). Most cells were either labeled and SA-β-Gal negative or unlabeled and SA-β-Gal positive. Double positive cells comprised <0.1% of early, and 5–8% of late, passage cultures. Such cells may have completed their last cell cycle and induced SA-β-Gal during the 3-day labeling or induced SA-β-Gal before the last cell cycle. Double negatives comprised 1–2% of early, and 20–25% of late, passage cultures. Some were slow-cycling cells, because labeling for 5–7 days reduced them from 22% to 16% and increased labeled cells from 15% to 20%. Others expressed low SA-β-Gal, since longer staining (24 vs. 8 hr) decreased them from 23% to 17% and increased SA-β-Gal positives from 69% to 75%. Ten to 15% of cells in senescent cultures were unlabeled and SA-β-Gal negative for unknown reasons. (*A* and *B*) Early passage (*A*) and senescent (*B*) WI-38 cells stained for lysosomal β-Gal. (*C*) Early passage HCA2 cells; labeled, SA-β-Gal staining. An unlabeled SA-β-Gal-positive cell is in the lower right. (*D*) Senescent HCA2; labeled, SA-β-Gal staining. A labeled SA-β-Gal-negative cell is in upper left. (*E*) Presenescent, confluent WI-38; SA-β-Gal staining. Staining disappeared 2 days after replating. (*F*) Early passage NHEK; labeled, SA-β-Gal staining. (*G*) Middle passage NHEK; labeled, SA-β-Gal staining. (*H*) Early passage differentiated NHEK; labeled, SA-β-Gal staining. (*I*) CMV-MJ cells 10 passages after receiving human chromosome 1; labeled, SA-β-Gal staining. Unlabeled SA-β-Gal-positive cells were not seen in the parent culture. (*J*) IDH4 cells growing in dex; SA-β-Gal staining. (*K*) IDH4 minus dex for 17 days; SA-β-Gal staining. (*L*) IDH4 minus dex for 20 days, then plus dex for 3 days; SA-β-Gal staining.

SA-β-Gal Is Not Induced Quiescence or Terminal Differentiation. Presenescent fibroblasts were made quiescent by

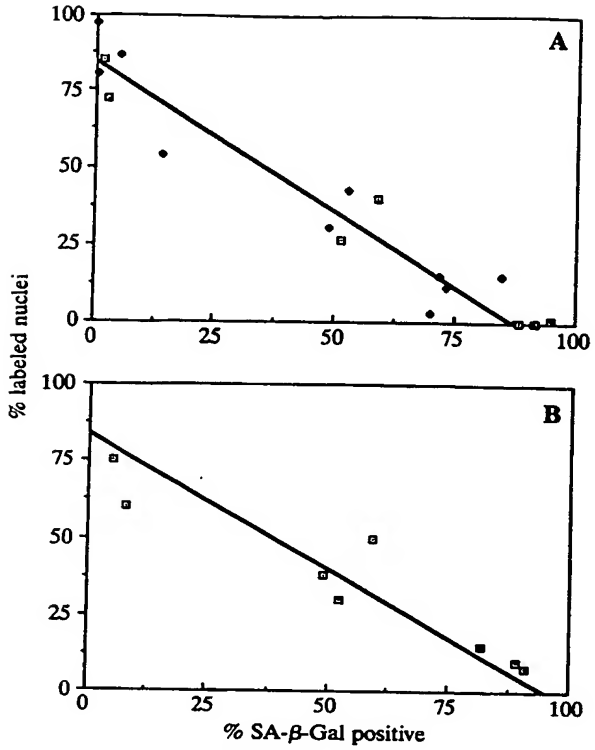

FIG. 2. SA-β-Gal and replicative capacity in culture. Cells were radiolabeled for 72 hr and stained, and 100–500 cells were scored for labeled nuclei and SA-β-Gal staining. (*A*) SA-β-Gal and replicative capacity during serial passage. Open symbols, WI-38 cells; closed symbols, HCA2 cells. (*B*) SA-β-Gal, replicative capacity, and donor age. The name, genotype, donor age, and percent labeled nuclei of five normal (N) and three Werner (W) skin fibroblast cultures are as follows: AG07720, N, 24 yr, 75%; AG0439, N, 73 yr, 30%; AG06234, N, 17 yr, 15%; AG09602, N, 92 yr, 60%; 88-6, N, 52 yr, 10%; AG00780, W, 59 yr, 50%; AG06300, W, 36 yr, 38%; MC17326, W, 57 yr, 8%.

serum deprivation. Growth declined markedly over 3 days, but SA-β-Gal was not induced, even after 7 days (Table 1). Similar results were obtained with cells made quiescent by confluence, except that positive staining was evident where cells were dense and overlapped (Fig. 1*E*). This staining was generally less intense than that seen in senescent cells and was lost within 2 days of replating.

Early passage human keratinocytes (NHEK) were also largely SA-β-Gal negative (Fig. 1*F*; Table 1) but stained for lysosomal β-Gal (not shown). After 8–10 PD, [^{3}H]thymidine labeling declined, and unlabeled SA-β-Gal-positive cells rose (Fig. 1*G*; Table 1). Thus, senescence and SA-β-Gal were also linked in keratinocytes. Moreover, early passage terminally differentiated NHEK did not express SA-β-Gal, despite a decline in proliferation (Fig. 1*H*; Table 1).

SA-β-Gal Correlates with Replicative Age. Skin fibroblasts from two young and two old adult donors were tested for SA-β-gal and replicative capacity. Each culture had undergone 15–20 PD when tested. Two had replicative potentials typical of their age (9–11), and two were unusual in this regard. In all cases, SA-β-Gal varied inversely with replicative age, not donor age (Fig. 2*B*). We also tested skin fibroblasts from donors with the Werner syndrome, a hereditary disorder showing accelerated aging *in vivo* and accelerated senescence in culture (13, 14, 26). Werner syndrome cultures accumulated SA-β-Gal-positive cells coincident with the loss of replicative capacity (Fig. 2*B*). Thus, SA-β-Gal reflected the replicative or physiologic age of cells, not necessarily the chronological age of the donor.

SA-β-Gal in Other Cell Types. SA-β-Gal and replicative senescence were correlated in several other cell types. Human umbilical vein endothelial cultures at early passage (65% labeled) had few SA-β-Gal-positive cells (11%), but positive cells increased (32% and 45%) at later passages (36% and 24% labeled). SA-β-Gal also correlated with replicative capacity in human mammary epithelial cells, rising from 2% at early passage to 30% at middle passage. Finally, neonatal human melanocytes were <1% SA-β-Gal positive at early passage but >90% positive at senescence. An exception was melanocytes from adult donors. Early passage adult melanocytes grew well in culture, yet >90% expressed pH 6 β-Gal activity.

Immortal Cells Do Not Express SA-β-Gal. SA-β-Gal was undetectable in immortal cells, including large tumor antigen (T-antigen)-immortalized WI-38 cells and human carcinoma, fibrosarcoma, and osteosarcoma cells (HT1080, HeLa, C33a, U2OS, SAOS, TE85, HTB9, CMV-MJ). These cultures were >90% [^{3}H]thymidine labeled and <0.1% SA-β-Gal positive.

Genetic manipulations that induced immortal cells to senesce induced SA-β-Gal. Human chromosome 1 causes CMV-MJ cells to senesce after 5–10 PD (17). Eight PD after chromosome 1 was introduced into these cells, 39% were SA-β-Gal positive and mostly unlabeled (Fig. 1*I*). Similar results were obtained with HeLa cells, in which chromosome 4 causes senescence after very few doublings (16). Virtually all cells in hybrid clones of <60 cells were SA-β-Gal positive. Chromosome 11 does not cause HeLa to senesce and did not induce SA-β-Gal (not shown).

Simian virus 40 (SV40) T antigen extends replicative lifespan (4, 8, 21) and delayed SA-β-Gal induction. Human ovarian epithelial cells senesce after 6–8 passages, but SV40-infected cultures proliferate for 20 passages (23). Normal cultures at fourth passage were 20% [^{3}H]thymidine labeled and 80% (mostly unlabeled) SA-β-Gal positive; fourth passage SV40-infected cultures were 80% labeled and 1% SA-β-Gal positive (not shown). T-antigen-expressing cultures eventually enter crisis, from which rare immortal cells emerge (8, 19). Less than 1% of SV40-infected human fibroblasts expressed SA-β-Gal prior to crisis, 95% were positive during crisis, and <0.1% were positive after immortal cells overgrew the culture (not shown). SA-β-Gal was also examined in IDH4 human fibroblasts, in which a dex-inducible T antigen reversibly regulates senescence (19). IDH4 cells growing in dex were <0.1% SA-β-Gal positive. After dex withdrawal, growth ceased and 88% of the cells expressed SA-β-Gal. SA-β-Gal

Table 1. Sa-β-Gal in senescence, quiescence, and terminal differentiation

Cells	Passage condition	% labeled nuclei	% SA-β-Gal positive
WI-38	Early/prolif	72	3
	Early/quies, 3 days	9	2
	Early/quies, 7 days	2	2
	Middle-late/prolif	27	52
	Middle-late/quies, 4 days	1	56
NHEK	Early/prolif	77	<0.1
	Early/diff (Ca, 4 days)	28	<0.1
	Early/diff (PMA, 4 days)	21	3
	Middle/prolif	53	23
	Middle/diff (Ca, 5 days)	12	2

WI-38 cells growing in 10% serum (prolif) were radiolabeled for 72 hr and stained for SA-β-Gal or were shifted to 0.2% serum for 3 or 7 days (quies) and stained, and parallel cultures were labeled for 48 hr thereafter. NHEK in growth medium (prolif) were similarly labeled and stained or were given 2 mM $CaCl_2$ (Ca) or 80 nM phorbol 12-myristate 13-acetate (PMA) for 4 or 5 days (diff), labeled for the last 48 hr, and stained. For each determination, 100–500 cells were counted.

declined to <0.1% when dex was resupplied and growth resumed (Fig. 1 *J–L*).

SA-β-Gal in Human Skin. To explore the idea that senescent cells accumulate *in vivo*, skin samples from 20 human donors, aged 20–90 yr, were sectioned and stained for β-Gal. Most cells in the sections stained well for lysosomal β-Gal (not shown). SA-β-Gal-stained sections were examined blind by a dermatological pathologist for the staining frequency and identity of positive cells (Fig. 3; Table 2). Two staining patterns were evident.

First, positive staining was always seen in the hair follicles, and frequently in associated sebaceous glands (Fig. 3*A*), and in eccrine glands and ducts (Fig. 3*B*). This staining was independent of donor age. A second pattern was seen in the dermis and epidermis (Fig. 3 *C–H*). This staining increased with age in frequency and intensity (Table 2).

In the dermis, SA-β-Gal-positive cells were tentatively identified as fibroblasts, and they were sparsely and randomly distributed. None of the young donors (<40 yr) showed dermal staining. By contrast, all but one old (>69 yr) donor had positive dermal staining (Table 2). In the epidermis, SA-β-Gal-positive cells were in the basal layers, randomly distributed in some sections, clustered in others. The upper differentiated layers were negative. About half of the young donors showed some epidermal staining, typically in one or a few cells. One young donor sample was from obviously sun-damaged skin yet was devoid of staining (Fig. 3*D*). By contrast, SA-β-Gal-positive cells were always seen in the epidermis of old donors. Two old donors had staining in isolated cells; the remainder had clusters of positive cells (Fig. 3 *G* and *H*).

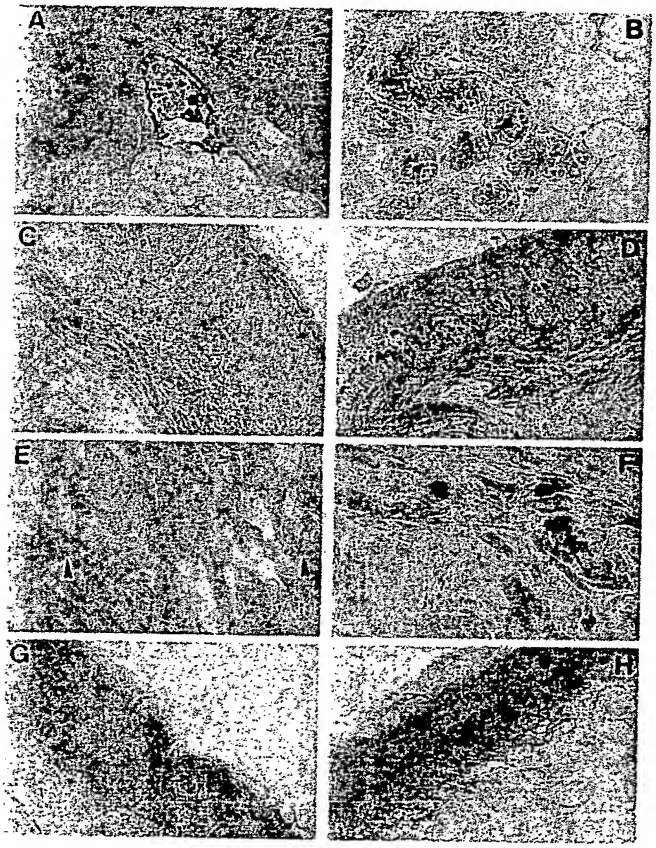

FIG. 3. SA-β-Gal in human skin. Human skin samples were sectioned, stained for SA-β-Gal, counterstained, and photographed at 100–400× magnification (final magnification given below). Sections were scored blind for SA-β-Gal frequency (Table 1). Scores for the sections shown are given in parentheses. (*A*) Age-independent staining in hair follicle and sebaceous gland. (×60; 38-yr-old female.) Note dense dermal collagen typical of young skin and absence of staining outside the follicle. (*B*) Age-independent staining in eccrine gland. (×120; 76-yr-old male.) (*C*) Young dermis and epidermis. (−, ×120; 38-yr-old female.) (*D*) Young dermis and epidermis. (−, ×120; 38-yr-old female.) Note sun damage (basal epidermal cell melanization) but absence of staining. (*E*) Old dermis. (++, ×120; 70-yr-old male.) Staining in single cells, indicated by arrowheads, was present in adjacent sections. (*F*) Old dermis. (+++, ×240; 76-yr-old male.) (*G*) Old epidermis. (++, ×120; 73-yr-old female.) (*H*) Old epidermis. (+++, ×120; 73-yr-old male.)

Table 2. SA-β-Gal activity in human skin from donors of different age

Donor	Age, yr	Sex	Site	SA-β-Gal staining: Epidermis	SA-β-Gal staining: Dermis
Young					
1	37	♀	Cheek	+	−
2	38	♀	Shoulder	−	−
3	37	♀	Nose	±	−
4	20	♀	Lip	−	−
5	39	♀	Lip	++	−
6	31	♂	Scalp	−	−
7	31	♀	Scalp	−	−
8	38	♂	Cheek	±	−
9	33	♂	Nose	+	−
10	31	♂	Temple	±	−
Old					
1	78	♀	Nose	+++	++
2	69	♂	Temple	±	++
3	73	♀	Nose	++	++
4	76	♂	Arm	+++	+++
5	81	♀	Lip	+++	−
6	70	♀	Lip	+++	++
7	81	♂	Neck	+++	+++
8	73	♂	Temple	+++	+++
9	75	♂	Scalp	++	+++
10	90	♂	Scalp	±	+++

Human skin samples from the indicated donors and sites were sectioned and stained. For each sample, at least five sections were viewed in a blinded fashion and assessed for SA-β-Gal. −, No staining; ±, one positive cell; +, two to four positive cells; ++, several positive cells in the dermis, clusters in the epidermis; +++, positive cells in all sections of dermis, multiple clusters in all sections of epidermis (see Fig. 3).

DISCUSSION

SA-β-Gal and Senescence. Cells from all three embryonic layers expressed SA-β-Gal upon senescence in culture. Still, some cells—adult melanocytes and sebaceous and eccrine gland cells—expressed the activity independent of senescence or age. In addition, we have not detected SA-β-Gal in senescent fibroblasts from two mouse strains, although senescent rat fibroblasts were positive. Thus, SA-β-Gal is not a universal marker of replicative senescence, which is not surprising. The age- or senescence-independent expression by some cells suggests that induction in otherwise nonexpressing cells may reflect senescence-associated changes in differentiation (see ref. 4). SA-β-Gal must have cell-specific functions, and its induction upon senescence may be limited to certain cell types. Nonetheless, SA-β-Gal was a good marker of senescence in some human cells. Moreover, its induction depended on replicative or physiologic age, suggesting that it could serve as a biomarker of aging. We suggest that the age-dependent rise in SA-β-Gal in human skin reflects an accumulation of senescent fibroblasts and keratinocytes *in vivo*.

Origin and Function. We do not yet know the origin or function of SA-β-Gal. A neutral β-Gal activity has been described in mammalian tissues (27). Senescence might entail

the induction of this or a related enzyme. Alternatively, because some lysosome activities are elevated in senescent cells (28, 29), lysosomal β-Gal may increase such that its activity is detectable at pH 6. Senescent cells might also express the alternately spliced (short) form of lysosomal β-Gal (25), which may encode pH 6 activity, or localization might alter the lysosomal β-Gal pH optimum. Preliminary data (G.P.D., B. Huang, and J.C., unpublished) favor the second possibility. *In situ* staining, which is not quantitative, showed little difference between presenescent and senescent cells at pH 4, but soluble assays showed 5- to 10-fold more pH 4 activity in senescent cells. The pH vs. activity profile was too broad to discern a distinct pH 6 peak. Senescent cells also expressed 3- to 5-fold more lysosomal β-Gal mRNA. Finally, when the long-form, but not short-form, cDNA was overexpressed in SA-β-Gal-negative cells, staining was detectable at pH 6. These data are consistent with lysosomal β-Gal overexpression giving rise, at least in part, to the activity in senescent cells.

It is unlikely that SA-β-Gal causes the growth arrest of senescent cells. Indeed, cells positive for SA-β-Gal and [^{3}H]thymidine were consistently, albeit infrequently, observed. SA-β-Gal most likely reflects the change in cell function that invariably accompanies senescence.

Senescence Genes and Pathology. SA-β-Gal provides a simple assay for chromosomes that induce senescence and should facilitate the cloning of senescence genes. It also provides *in situ* evidence that senescent cells may accumulate *in vivo* at sites that show age-related pathology. Dermal thinning and collagen breakdown are hallmarks of aging skin that may be due to senescent fibroblasts, which overexpress collagenase and underexpress collagenase inhibitors (30). Similarly, endothelial cells lining vessels subject to hemodynamic stress may senesce early in life (C. Harley and M.L., unpublished). The altered functions of senescent endothelial cells (31) may initiate or exacerbate atherosclerosis. Further studies are now needed to determine how SA-β-Gal relates to other physiologic signs of age and to show that senescent cells indeed contribute to age-related pathology. A biomarker for replicative senescence should facilitate these studies.

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